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Involvement of host and bacterial factors in *Agrobacterium*-mediated transformation

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Chapter 4

Use of the Auxin-induced degron system to study the role of virulence protein VirD2 in the integration of T-DNA into the plant and yeast genome

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Abstract

Agrobacterium tumefaciens is the causative agent of crown gall, an important plant disease. It induces tumor formation by transferring a segment of its tumor-inducing plasmid (Ti-plasmid), the T-DNA, to plant cells. The virulence protein VirD2 plays a crucial role in many stages of the transfer process. It is not only essential for the formation of single-stranded T-DNA (T-strand), but also for the translocation of the T-strand through the Type IV secretion system (T4SS) into host cells. Inside host cells it supports the targeting of the T-complex into the nucleus and within the nucleus it may be involved in the integration of the T-DNA into the chromosomal DNA. However, whether VirD2 plays any role within the nucleus is still unclear. In this chapter we exploited the auxin-inducible degron system to investigate the importance of VirD2 within the nuclei of host cells for transformation. The results demonstrate that AMT efficiency is affected by the degradation of VirD2 within the nuclei of both plant and yeast cells. Stable transformation was strongly affected, but transient expression relying on the delivery into the nucleus was much less affected suggesting a role of VirD2 in the targeting to chromatin or in T-DNA integration.

Introduction

Agrobacterium tumefaciens, a gram-negative plant pathogen belonging to the family *Rhizobiaceae*, is the causative agent of crown gall disease, which causes severe damage to worldwide agriculture. It induces tumor formation in plants by transferring a segment of its tumor-inducing plasmid (Ti-plasmid) to plant cells. This transferred DNA (T-DNA) contains genes encoding enzymes involved in the synthesis of auxin, cytokinin and opines resulting in uncontrolled cell proliferation and production of opines. Tumorigenic genes within the T-DNA segment can be deleted, to disarm the plasmid, and be replaced with foreign DNA which then instead becomes integrated into the plant genome as part of the infection process. Under laboratory conditions *A. tumefaciens* is also able to transform other eukaryotes such as yeast and fungi (Bundock et al. 1995; Lacroix et al. 2006). Hence, *A. tumefaciens* has been extensively used as a vector not only to create transgenic plants, but also for fungal transformation and over the past decades *Agrobacterium*-mediated transformation (AMT) has become the preferred method of transformation of many of these organisms (reviewed by Nester et al., 1984; Gelvin, 2003; Tzfira and Citovsky, 2006; Păcurar et al., 2011; Christie and Gordon, 2014; Gelvin, 2017).

AMT can be depicted as a process with multiple stages (reviewed by Gelvin, 2017). First of all, several plant chemicals, such as phenolic compounds along with neutral and acidic sugars, which are produced at the wound sites of plant tissues, trigger a two-component sensory-response system. This system is composed of the *Agrobacterium* VirA and VirG proteins and upon stimulation it will lead to the expression of virulence genes. The virulence protein VirD2, together with VirD1, nicks at 25-bp direct repeat sequences surrounding the T-DNA called left border (LB) and right border (RB) to generate a single stranded copy of this DNA segment (T-strand). VirD2 remains covalently bound to the 5' end of the T-strand at the RB. Simultaneously, a variety of virulence proteins are expressed. The VirB1-11 and VirD4 proteins form a dedicated secretion channel (Type IV secretion system, T4SS) to translocate the T-strand-VirD2 complex along with other effector proteins, including VirE2, VirE3, VirD5 and VirF, to the host cell. Inside the host cell, VirE2 is thought to coat the T-strand to prevent digestion by host nucleases and may target together with VirD2 the T-strand into the nucleus. The VirD5 protein can cause chromosome instability, while VirF may contribute to the removal of VirE2 proteins bound to the T-strand and VirE3 acts as a transcription factor in plant cells. Eventually, the T-DNA will integrate into the host genome mainly through host specific DNA repair mechanisms (reviewed by Tzfira and Citovsky, 2006; Păcurar et al., 2011; Gordon and Christie, 2014; Gelvin, 2017).

VirD2 plays a crucial role in several steps of the AMT process. The N-terminal region of VirD2 has endonuclease activity, nicking at the borders of the T-DNA to release the T-strand (Yanofsky et al., 1986; Young and Nester, 1988). The T-strand remains covalently bound to VirD2 and VirD2 helps in the translocation of the T-strand through the T4SS into the host cell (Vergunst et al., 2005; van Kregten et al., 2009). The nuclear localization signal sequence (NLS) present in the C-terminal part of VirD2 interacts with importin α and thus contributes to targeting the T-complex to the host nucleus (Howard et al., 1992). It may even be involved in the integration of the T-DNA into the chromosomal DNA. However, the role of VirD2 in the integration of the T-DNA into the host chromosomal DNA remains obscure and in dispute. An aberrant integration pattern caused by mutated VirD2 protein was revealed, thus indicating an

important role of VirD2 in the precise integration of T-DNA into plant genome (Tinland et al., 1995). Later, by a ligation-integration assay *in vitro*, VirD2 was found not to possess general ligase activity, which argued against a function of VirD2 as an integrase and ligase in T-DNA integration (Ziemienowicz et al., 2000). Moreover, plant enzymes were found to mediate T-DNA ligation *in vitro*. VirD2 interacts in plant and yeast cells with proteins important for DNA and histone modification. The S-adenosyl-L-homocysteine hydrolase (involved in DNA methylation) and a MYST-like histone acetyltransferase 2 were reported to interact with VirD2 (Lee et al., 2012). Also it was found that VirD2 can bind to histone proteins in the yeast *Saccharomyces cerevisiae* (Wolterink-van Loo et al., 2015). In addition, VirD2 can mediate ligation of T-DNA border sequences to themselves by a strand transferase reaction, effectively reversing the original nicking reaction (Pansegrau et al., 1993) and thus may contribute to T-DNA circularization in host cells.

To gain more insight into the role of VirD2 in host cells during AMT, conditional inactivation or depletion of VirD2 may be a powerful method. The described auxin-inducible degron (AID) system, using a plant hormone-induced degradation signal, has successfully been used to control protein levels in yeast (Nishimura et al., 2009). In its natural context, auxin (indole-3-acetic acid; IAA) induces degradation of the IAA proteins, a family of short-lived transcriptional repressors, by mediating the interaction of a degron domain in the target protein with the substrate recognition domain of an F-box protein, TIR1, which forms part of a SCF-type ubiquitin ligase (E3). Interaction in the presence of auxin leads to ubiquitylation of the target protein and proteasomal degradation. The SCF complex, consisting of a cullin subunit, a catalytic RING finger protein (RBX1), the adaptor SKP1 and an F-box protein as a substrate recognition subunit, is highly conserved among eukaryotes. Therefore, the plant F-box protein TIR1 is able to form an active E3 complex with the remaining SCF components from other organisms. Hence, constitutive expression of TIR1 allows a reconstitution of the AID system in yeast (Morawska and Ulrich, 2013) or mammalian cells (Holland et al., 2012). Proteins of interest are fused to an auxin-dependent degron sequence derived from IAA17. This AID tag can in principle be placed at the N- or C-terminus of the target protein, thus making the system more flexible than the N-end rule degron. Degradation is reversible and quick, active in minutes rather than in hours. Furthermore, due to the lack of an auxin-responsive system in animals or yeast, the hormone as well as the F-box protein are otherwise biologically silent and cause no measurable physiological changes in the absence of a target, thus minimizing possible side-effects of the treatment (Nishimura et al., 2009).

The TIR1 protein is localized to the nucleus in plant cells (Dharmasiri et al., 2005). This allowed us to exploit the system to specifically target VirD2 for degradation when it entered the nucleus and to study whether this degradation would affect AMT. The results presented here support the idea that VirD2 participates in processes inside the host nucleus that are important for transformation.

Methods

Bacterial cells and growth conditions

Agrobacterium tumefaciens strains used in this chapter are listed in Table 1. Plasmids were transferred into *Agrobacterium* by electroporation (den Dulk-Ras and Hooykaas, 1995). *A. tumefaciens* strains were grown and maintained at 29°C in LC medium (10 g/L tryptone, 5 g/L

yeast extract and 8 g/L NaCl) containing appropriate antibiotics such as carbenicillin (100 µg/mL), kanamycin (100 µg/mL) or rifampicin (20 µg/mL) when required. *Escherichia coli* DH10B was used for plasmid amplification.

Yeast strains and growth conditions

S. cerevisiae strains used in this study can be found in Table 1. DF5-TIR1 *ada2Δ* was constructed using a standard PCR-mediated one-step gene disruption protocol (Kaiser et al., 1994). The disruption cassette was amplified with the primers ADA2-Fw/Rev from plasmid pAG32 (as mentioned in Chapter 2). Yeast was grown at 30°C in yeast extract-peptone-dextrose (YPD) medium supplemented, when required, with the antibiotic G418 (200 µg/mL) or hygromycin (200 µg/mL) or in selective minimal yeast (MY) (Zonneveld, 1986) medium supplemented with appropriate nutrients. Plasmids were transferred to yeast cells using the lithium-acetate transformation protocol (Gietz et al., 1995)

Plasmid constructions

All plasmids and primers used in this chapter are listed in Table 2 and Table 3, respectively. The newly constructed plasmids were checked by restriction digestion and sequence analysis.

Plasmids used in the auxin-induced degron system were constructed as follows. Initially, a fragment with the C-terminal part (AID⁷¹⁻¹¹⁴) of IAA17 was obtained by PCR with the primers AID-Fw/Rev using pHyg-AID¹⁻¹¹⁴-8myc as template. This fragment was digested with *KpnI* and *XmaI* and cloned into pHyg-AID¹⁻¹¹⁴-8myc digested with the same enzymes to replace IAA¹⁻¹¹⁴ forming plasmid pSDM4694. Subsequently, the C terminal T4SS translocation signal of protein VirF (VirFCT) was amplified from vector pGPINTAM (Friml et al., 2004) with the primers VirFCT-Fw/Rev and this VirFCT fragment was cloned into the *XmaI* site of pSDM4694 and pSDM4697, resulting in pSDM4694(VirFCT) and pSDM4697(VirFCT), respectively. Finally, two fragments, IAA¹⁻¹¹⁴-VirFCT and AID⁷¹⁻¹¹⁴-VirFCT, were generated by PCR with the primers IAA-Fw/Rev for cloning (see below).

The *virD2* gene, including its promoter region, but lacking its last 3 codons and the stop codon, was amplified by PCR using pSDM3149 as template with the primers VirD2-Fw/Rev and then cloned as *XbaI/EcoRI* fragment into plasmid pSDM3149 to form plasmid pSDM4698 (VirD2-no stop codon). The fragment containing IAA¹⁻¹¹⁴-VirFCT or AID⁷¹⁻¹¹⁴-VirFCT signals were cloned into the *EcoRI* site of pSDM4698, resulting in the plasmids pSDM4699 (VirD2^{AID}) and pSDM4700 (VirD2^{IAA}), respectively. Simultaneously, in order to monitor the degradation of VirD2 by using microscopy, the same fragments were cloned into the *EcoRI* site of plasmid pUG34-virD2 (pRUL1146) (Soltani, 2009), to form plasmids pSDM4696 (VirD2^{AID}) and pSDM4695 (VirD2^{IAA}), respectively.

Tumor assay

Four-week old *Nicotiana glauca* and *Kalanchoe tubiflora* were wounded with a sterile syringe at three series sites on the stem. *A. tumefaciens* cells were cultured overnight at 28°C with the appropriate antibiotics and then washed three times and resuspended in 0.9% (w/v) NaCl to an A₆₀₀ of 1.0. Subsequently, *N. glauca* and *K. tubiflora* were injected with 20 µl of *A. tumefaciens* cells at the wound sites and tumors were photographed after 3–4 weeks.

Root transformation and GUS assays

Root transformation was performed as described (Vergunst et al., 2000). Root segments from *Arabidopsis thaliana* were infected with *A. tumefaciens* LBA1100 harboring the binary vector pCambia2301. After co-cultivation on callus induction medium containing 100 μ M acetosyringone for 48 hours, root segments were washed, dried and incubated on plates containing shoot induction medium with 30 μ g/ml phosphinothricin, 500 μ g/ml carbenicillin and 100 μ g/ml vancomycin. After 3-4 weeks, plates were photographed and callus formation was scored to calculate transformation efficiencies.

For transient GUS activity assays (Jefferson et al., 1987), *Nicotiana benthamiana* leaves and *A. thaliana* roots were co-cultivated for 3 days and then washed and placed in a 6-well microtiter plate. The wells were sealed with tinfoil. The leaf and root segments were incubated with X-Gluc overnight at 37°C and destained by washing three times with 70% ethanol. After that the GUS staining was examined under a Leica MZ 12 microscope (Leica microsystems). The leaves were photographed with a Leica DC 500 digital camera (Leica microsystems) and the blue spots on the roots were counted.

Agrobacterium mediated transformation of yeast

AMT efficiency was determined as described by Bundock et al. (1995) with some modifications. Firstly, *S. cerevisiae* and *Agrobacterium* strains were grown overnight at 30°C and 28°C, respectively, under continuous shaking and with the appropriate antibiotic selection. The following day, the *Agrobacterium* culture was centrifuged, the cells were washed with induction medium (IM) and re-suspended to an A_{620} of 0.25 in IM with added glucose (10 mM), acetosyringone (AS, 0.2 mM) and appropriate antibiotics, and incubated for another 6 hours at 28°C. Meanwhile, yeast overnight cultures were diluted to an A_{620} of 0.1 and incubated again in either liquid YPD or MY medium for 6 hours. Then, yeast cultures were centrifuged and cells were washed with IM and re-suspended in 0.5 ml of IM to a final A_{620} of 0.4 - 0.6 and mixed by vigorous vortexing with an equal volume of *Agrobacterium* cells. Subsequently, 100 μ l of the mixture were pipetted onto sterile nitrocellulose filters laid on IM plates supplemented with histidine, leucine, uracil and methionine. Once the filters were dry, plates were incubated at 21 °C for 6-7 days. After co-cultivation, the cell mixture was washed off the filters and then spread onto YPD plates containing cefotaxime (200 μ g/mL) with or without G418 (200 μ g/ml). Finally, after a 3-days incubation at 30 °C, colonies were counted. Yeast AMT efficiency was calculated by dividing the number of colonies on the selective plates by the number of colonies on the non-selective plates.

Confocal microscopy and Flow Cytometry

Yeast cells were co-cultivated for 2-3 days with *Agrobacterium*, then washed off the filters with MY medium and an aliquot was analyzed by confocal microscopy using a 63x oil objective on the Zeiss Imager M1 confocal microscope equipped with a LSM5 Exciter. GFP was detected using an argon laser of 488 nm and a band-pass emission filter of 505-600 nm. Images were processed with ImageJ (ImageJ National Institute of Health) (Schindelin et al., 2012).

For flow cytometry all yeast strains were grown in MY medium supplemented with the appropriate nutrients. Cultures were diluted 10-fold before flow cytometry and measured by a Guava easyCyte™ system (Merck Millipore). Data were analyzed with CytoSoft software and in each experiment, 5000 cells were analyzed.

Statistical analysis

All data shown are representative of at least 3 independent experiments of which each contains at least 3 biological replicates and represented as mean of the performed experiments with standard deviation. Statistical tests were done with two-tailed Student's t-test. Statistical analyses were performed using Microsoft Excel. An asterisk indicates a significant differences with a P-value <0.05.

Results

AID degron system allows VirD2 protein degradation in yeast

In order to get a better understanding of the role of VirD2 in the integration process during AMT, we exploited the auxin-inducible degron system to manipulate the level of the VirD2 protein (Figure 1).

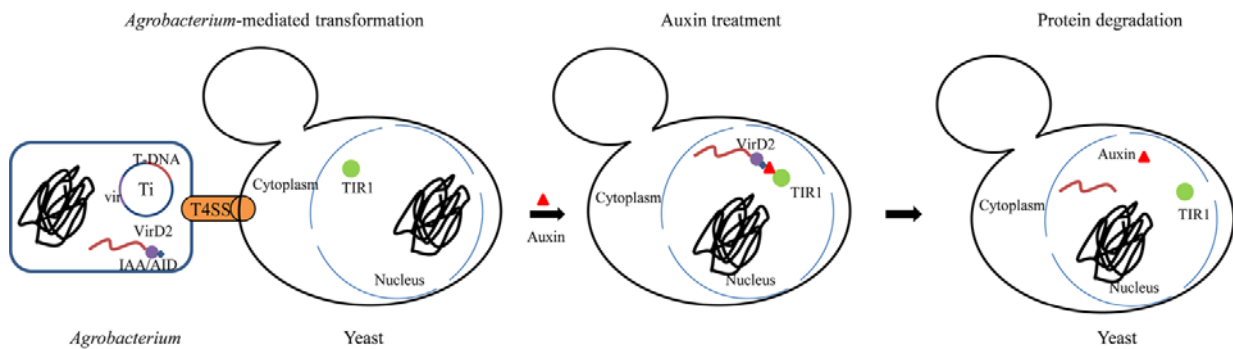
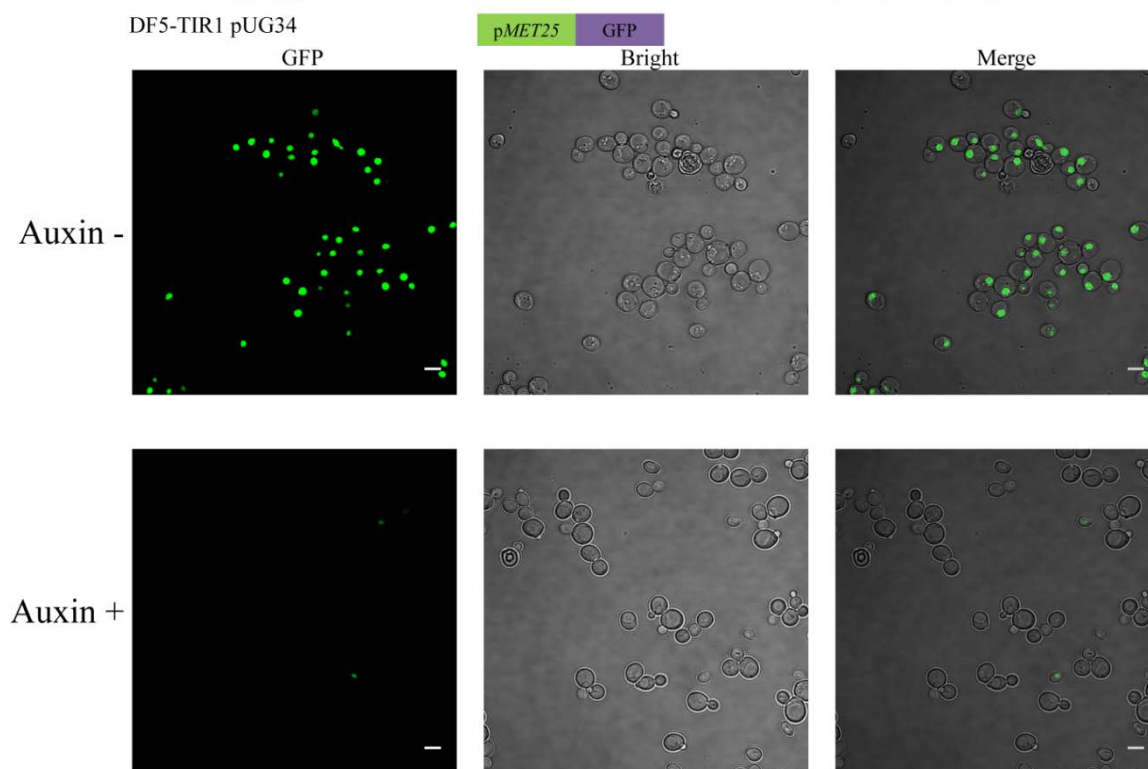
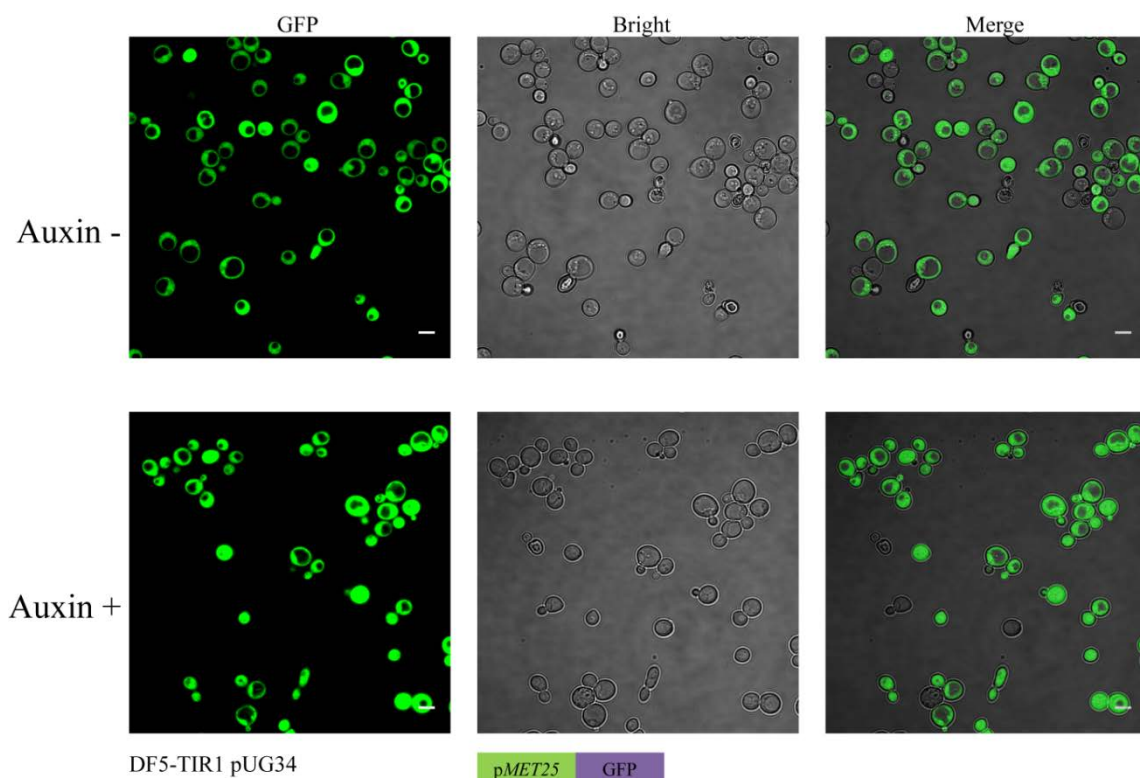


Figure 1. Schematic illustration of the AID system used to study the role of VirD2 inside yeast in AMT. From left to right: the modified VirD2 is bound to the T-strand at its RB and guides the T-strand through the T4SS into the yeast cell; upon translocation of the T-complex into the nucleus addition of auxin promotes the interaction between TIR1 and the AID degron signal (IAA/AID) of the modified VirD2 protein; finally, the modified VirD2 is subject to proteasomal degradation. Purple dot, VirD2; blue diamond, IAA/AID sequence; green dot, TIR1.

This system relies on the F-box protein Tir1, which is located in the nucleus of plant cells (Dharmasiri et al., 2005; Bian et al., 2012). To this end we tagged the VirD2 protein with the IAA17 protein resulting in VirD2^{IAA} containing amino acids 1-114 of IAA17 or with an N-terminal truncation of IAA17 resulting in VirD2^{AID} containing amino acids 71-114 of IAA17 as described (Morawska and Ulrich, 2013). To restore the ability of VirD2 to translocate through the T4SS, we added the T4SS recognition sequence of VirF to the C-terminal ends. For our initial analysis, we tagged both modified VirD2 proteins with GFP (constructs shown at the bottom of Figure 2B and 2C). The fusion proteins were expressed in yeast strain DF5-TIR1 expressing TIR1-9Myc. As in plant cells TIR1 is localized in the nucleus, we assumed that TIR1-9Myc is also located in the nuclei of yeast cells. As demonstrated in Figure 2 (B and C), both GFP-VirD2^{IAA} and GFP-VirD2^{AID} accumulated in structures resembling the nucleus. In control strains expressing free GFP, fluorescence was found all over the cell (Figure 2A). These results are in agreement with previous research showing that ectopically expressed GFP-VirD2 is localized in the nucleus (Wolterink et al, 2015). Upon treatment with Auxin (IAA) the control GFP signals were unaffected (Figure 2A), while both GFP-VirD2^{IAA} and GFP-VirD2^{AID} became

Figure 2 (A and B). Degradation of modified VirD2 protein in yeast can be achieved with the auxin-induced degron system. Yeast cells DF5 stably expressing TIR1-9Myc were transformed with plasmid pUG34 encoding either free GFP (A) and GFP-VirD2^{IAA} (B) then they were treated with (+) or without (–) 1 mM Auxin (Indole-3-acetic acid) in an 1h incubation after cultivation overnight.

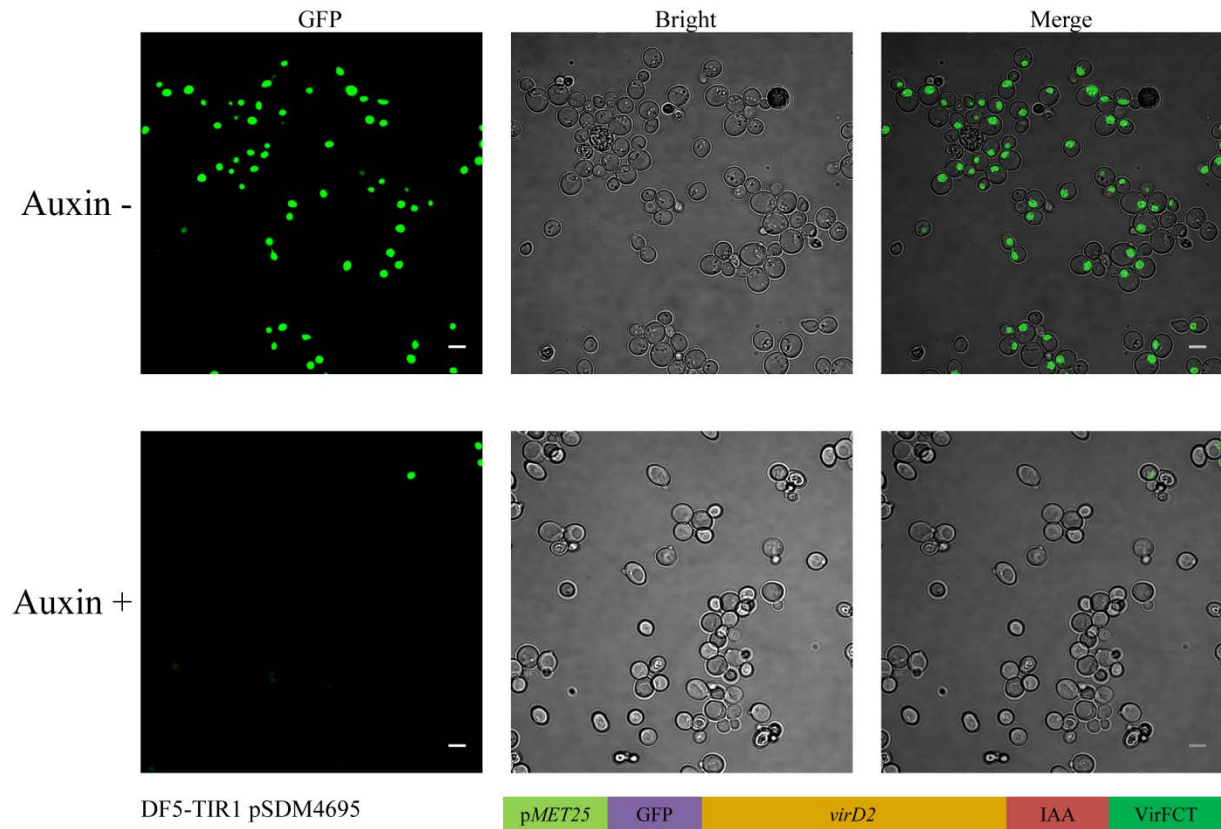
A.



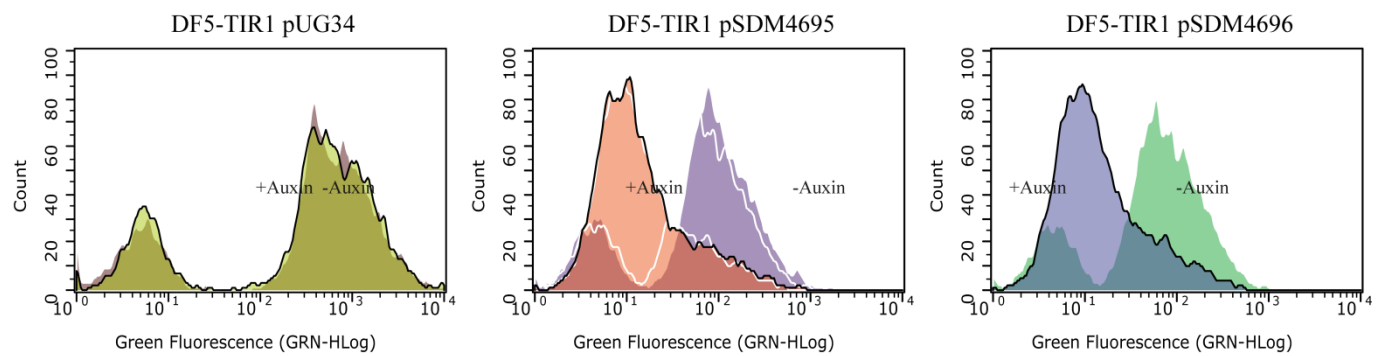
B.

Figure 2 (C and D). Degradation of modified VirD2 protein in yeast can be achieved with the auxin-induced degron system. Yeast cells DF5 stably expressing TIR1-9Myc were transformed with plasmid pUG34 encoding GFP-ViD2^{AID} (C). After cultivation overnight, they were treated with (+) or without (-) 1 mM Auxin (Indole-3-acetic acid) in an 1h incubation and cells were imaged by confocal microscopy and fluorescence was quantified by flow cytometry (D).

C.



D.



undetectable in most cells by microscopy revealing the presence of an active TIR1-SCF complex in the yeast nucleus (Figure 2B and 2C). The effect of Auxin treatment was further analyzed by flow cytometry. As illustrated in Figure 2D, a significant fluorescent signal could be detected in 95.4% and 95.8% of the cells expressing VirD2^{IAA} or VirD2^{AID}, respectively, but upon auxin treatment it could be detected in only 15.9% and 15.9% percent of the cells, respectively. Average fluorescence intensity was reduced by 72.5% (62.6 to 17.2, arbitrary units) and 67.0% (51.5 to 17.0, arbitrary units) for strains expressing VirD2^{IAA} or VirD2^{AID}, respectively. The levels of free GFP were not affected (Figure 2D). Significant fluorescent signals were detected in around 95% of the cells expressing free GFP when treated or not treated with auxin, while average fluorescence intensities were 268.7 and 272.1 (Arbitrary units), respectively. The flow cytometry experiment was performed twice and in both experiments the same trend was observed. Therefore, it can be concluded that both VirD2^{IAA} and VirD2^{AID} can be degraded by the auxin-inducible degron system within the nucleus. Because of its smaller size VirD2^{AID} will be used in our further research.

Targeted degradation of VirD2 in yeast affects AMT

To investigate the effect of degradation of VirD2 by the AID degron system on AMT, we introduced pBBR6[VirD2] or pBBR6[VirD2^{AID}] expressing native or AID-tagged VirD2, respectively, in the *Agrobacterium virD2* deletion mutant LBA2556. Subsequently, the binary vector pSDM8001 was introduced which allows T-DNA integration into the yeast *PDA1* locus resulting in G418 resistant yeast transformants. The constructed *Agrobacterium* strains were co-cultivated with yeast strain DF5-TIR, in which *TIR1* is expressed continuously and TIR1 binds to the AID-tag to degrade VirD2^{AID} in the presence of auxin. As shown in Figure 3A, in the absence of auxin both *Agrobacterium* strains expressing native VirD2 or AID-tagged VirD2 were able to transform DF5-TIR with a similar efficiency. The efficiency of VirD2^{AID} mutant was slightly lower than that of the VirD2 strain (1.2×10^{-4} vs 0.8×10^{-4}). On the other hand, in the presence of auxin, an approximately 10-fold decrease in the transformation efficiency of the *Agrobacterium* strain expressing VirD2^{AID} was found (0.1×10^{-4}), which was not observed for the *Agrobacterium* strain expressing native VirD2 (1.0×10^{-4}).

Considering the increased AMT efficiency of the *ada2A* mutant described in Chapter 2, we tested the possibility whether the enhanced AMT efficiency can be suppressed by the degradation of VirD2. To this end an *ada2A* deletion mutant was constructed in the DF5-TIR1 genetic background. This strain and the parent strain DF5-TIR1 were co-cultivated with *A. tumefaciens* strains expressing either VirD2 or VirD2^{AID}. As shown in Figure 3B (left part), in the presence of auxin the *ada2A* mutant is transformed with an higher efficiency (6.6×10^{-4}) compared to the parental strain DF5-TIR1 (3.7×10^{-4}) by the *Agrobacterium* strain expressing native VirD2 as observed previously in other strain backgrounds (Chapter 2). On the other hand, in the presence of auxin the transformation efficiencies by *Agrobacterium* expressing VirD2^{AID} were strongly reduced for both DF5-TIR1 and DF5-TIR1 *ada2A* strains (1.9×10^{-5} and 2.2×10^{-5} , respectively) (Figure 3B, right part). These results indicate that degradation of VirD2 also strongly reduces AMT efficiency of the *ada2A* strain.

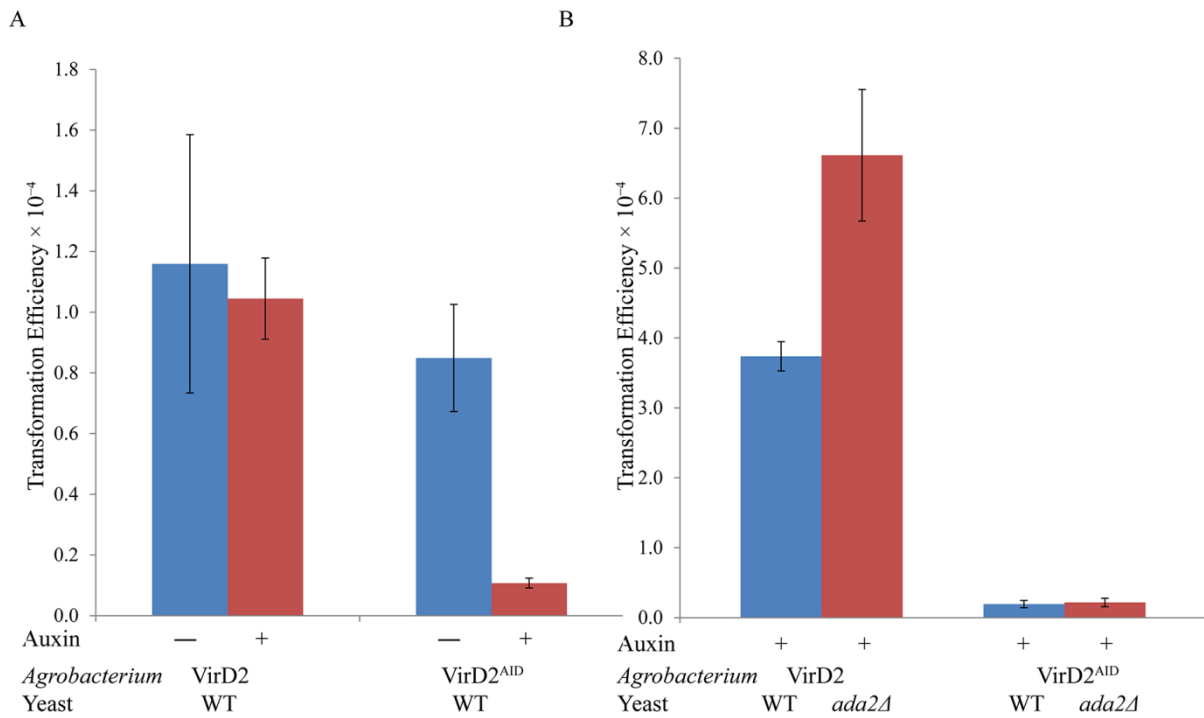


Figure 3. AID-mediated degradation of VirD2 affects AMT efficiency of yeast. (A) DF5-TIR1 yeast cells were co-cultivated for 7 days with *Agrobacterium* strain LBA2556-pSDM3149-pSDM8001 expressing native VirD2 or with LBA2556-pSDM4699-pSDM8001 expressing VirD2^{AID}. The induction medium was either supplemented or not supplemented with Auxin (Indole-3-acetic acid). Transformation efficiency was determined after selection in the presence or absence of G418. Error bars indicate the standard deviations of triplicate independent assays. (B) DF5-TIR1 and its *ada2Δ* mutant were co-cultivated in the presence of auxin with *Agrobacterium* strain LBA1100-pSDM8001 expressing native VirD2 or with LBA2556-pSDM4699-pSDM8001 expressing VirD2^{AID} for 7 days. Error bars indicate the standard deviations of triplicate independent assays.

Targeted degradation of VirD2 affects AMT of plants

In order to test the effect of the AID tag on tumor formation, *virD2^{AID}*, *virD2^{LAA}* and native *virD2* were cloned into the expression vector pBBR6, introduced in the *A. tumefaciens virD2* deletion mutant LBA2569 and tumor formation by these strains on *N. glauca* and *K. tubiflora* was analyzed. The wild type *A. tumefaciens* strain LBA1010 was included as a positive control. It is well known that auxin is present in all parts of the plant, although in different concentrations; therefore, addition of auxin is probably not necessary for VirD2^{AID} degradation in plant cells. As demonstrated in Figure 4A, the tumors on *N. glauca* caused by *Agrobacterium* strains LBA2569 expressing VirD2^{LAA} or VirD2^{AID} were much smaller than those caused by the wild type strain LBA1010, indicating an important role of VirD2 within the plant cell nucleus in transformation. In a second experiment tumor formation on both *N. glauca* and *K. tubiflora* was analyzed. As shown in Figure 4B (upper part) on both plant species tumors formed by *Agrobacterium* strain LBA2569 expressing VirD2^{AID} were smaller than tumors formed by LBA2569 expressing native VirD2. Injection of 100-fold less *Agrobacterium* cells resulted in smaller tumors for all strains (Figure 4B, lower part). The tumors formed after injection with *Agrobacterium* strain LBA2569 expressing VirD2^{AID} at OD₆₀₀ = 1 were similar in size to those formed by the *Agrobacterium*

strain expressing native VirD2, added at $OD_{600} = 0.01$. These results indicate that degradation of VirD2 within the plant cell nuclei strongly reduces the transformation efficiency.

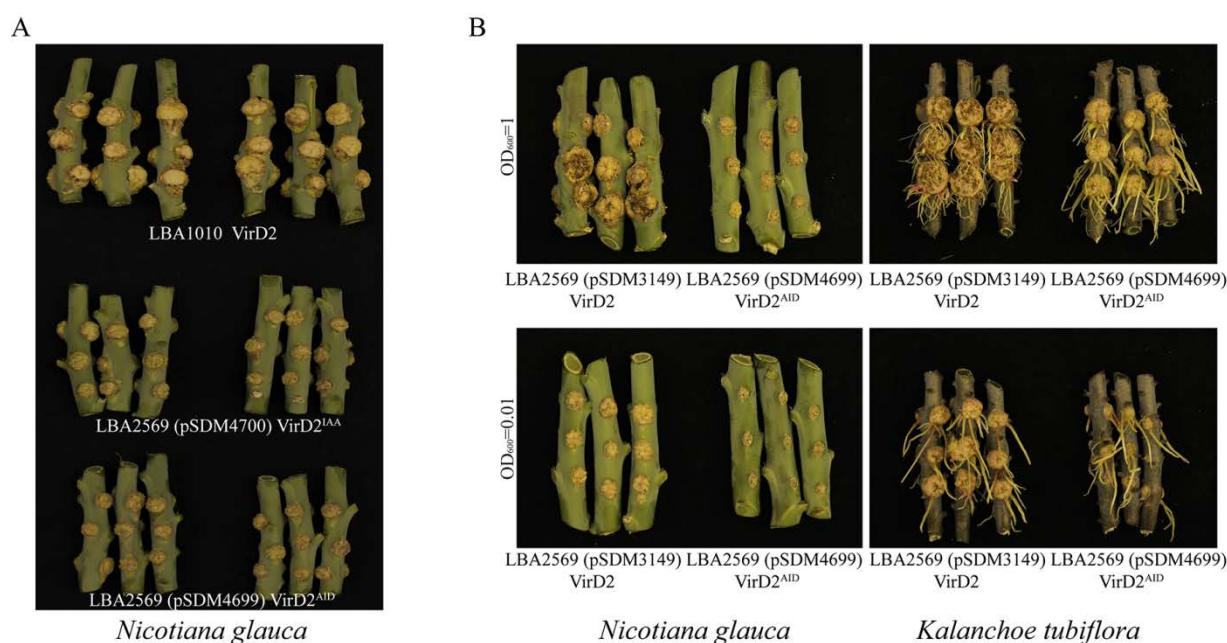


Figure 4. Tumor formation assay on plants. (A) *N. glauca* was infected with the *Agrobacterium* wild type strain LBA1010 and its *virD2A* derivative LBA2569 harboring plasmid pSDM4699 or pSDM4700 expressing VirD2^{AID} or VirD2^{IAA}, respectively. Tumors were photographed after 3 weeks. (B) *N. glauca* and *K. tubiflora* were injected with different initial concentrations ($OD_{600}=1$ or $OD_{600}=0.01$) of *Agrobacterium* strains LBA2569 harboring plasmid pSDM3149 or pSDM4699 expressing VirD2^{AID} or native VirD2, respectively. Tumors were photographed after 3 weeks.

In order to further investigate the effect of degradation of VirD2 by the AID system, we performed a quantitative transformation assay on *A. thaliana* roots. To this end, the binary vector pCambia2301 containing the kanamycin resistance gene and the *gus* reporter gene was introduced into *A. tumefaciens* strain LBA1100 expressing native VirD2 and into the LBA2556 expressing VirD2^{AID}. The formation of green calli was analyzed after four weeks (Figure 5). The number of calli formed by the strain expressing VirD2^{AID} was only 27% of the number of calli formed by the strain expressing native VirD2. This observation is in line with the results of the tumor formation assay and indicates that degradation of VirD2 within the nucleus of plant cells strongly and negatively affects the efficiency of AMT in plants.

T-DNA transfer is not affected by the degradation of VirD2

As shown in the previous section, degradation of VirD2 by the AID system, resulted in a lower transformation frequency in both yeast and plants. In order to investigate the effect of tagging VirD2 with AID on the transfer of the T-DNA from *Agrobacterium* into the host nuclei, we performed a transient transformation assay on *A. thaliana* roots using the same constructs as were used in the root transformation assay. Upon transfer of T-DNA from pCambia2301 into the plant cells, GUS activity can be detected independently of and prior to integration into one of the chromosomes.

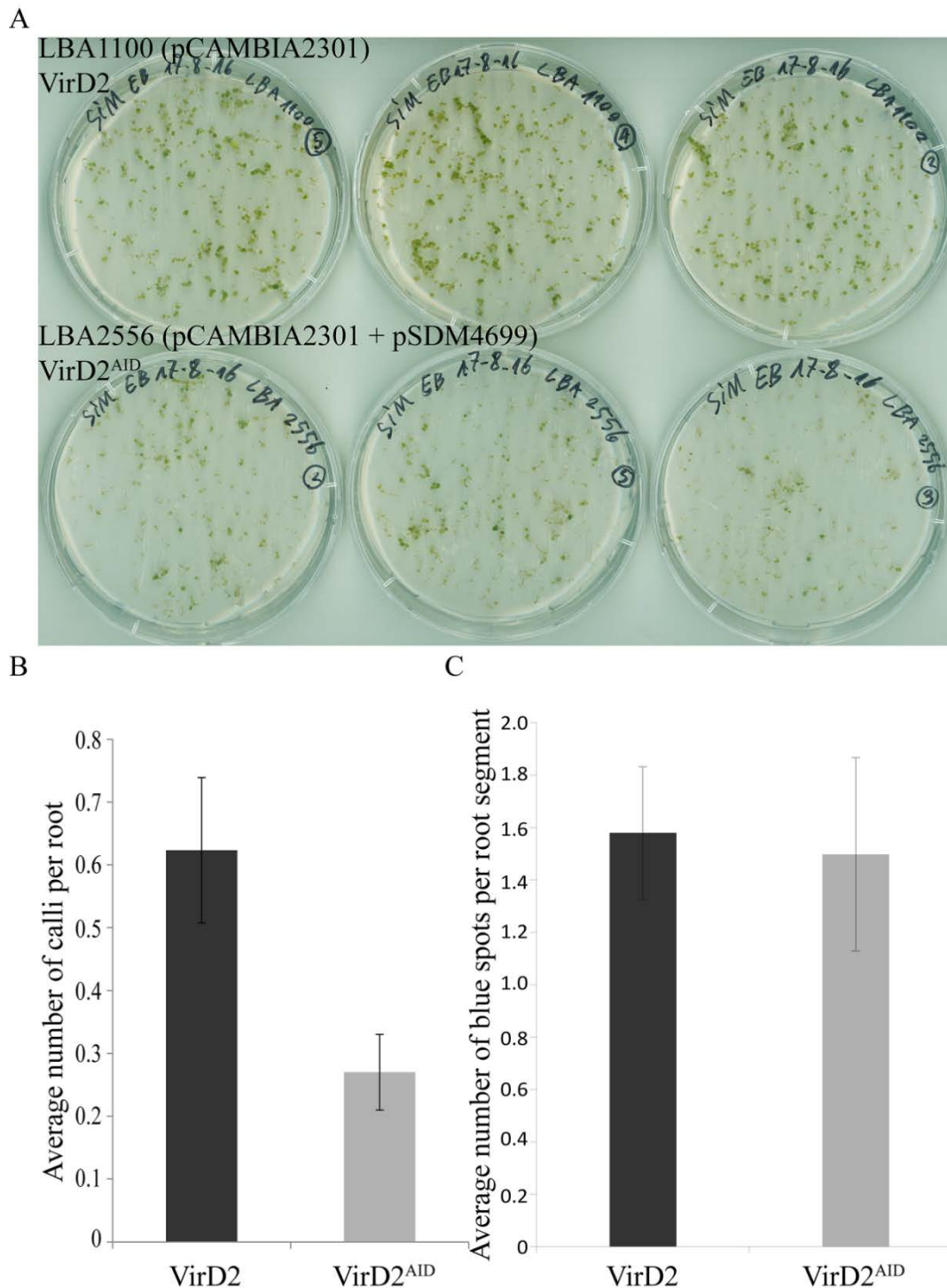


Figure 5. *Arabidopsis* root transformation assay to study the effect of AID-mediated degradation of VirD2 . *Arabidopsis* root segments were co-cultivated with *Agrobacterium* strain LBA1100-pCambia2301 expressing native VirD2 or LBA2556-pSDM4699-pCambia2301 expressing VirD2^{AID}. (A and B) Root segments were transferred to shoot induction medium containing kanamycin for selection of T-DNA integration. Root transformation was scored after 4 weeks. Efficiency is presented by the ratio of the average number of green calli per root segment relative to the total number of root segments. (C) Root segments were stained with X-Gluc. Blue spots of co-cultivated *A. thaliana* roots were counted and statistical analysis demonstrated no significant differences. Error bars indicate the standard deviations of triplicate independent assays.

After co-cultivation with *A. tumefaciens* strains for 3 days, the root segments were stained with X-Gluc to reveal transient transformation and the blue spots were quantified. As illustrated in Figure 5C no significant differences were observed in the numbers of blue spots seen after transfer from strains expressing VirD2 or VirD2^{AID}. This indicates that VirD2^{AID} is as active as native VirD2 in transferring T-DNA into the nuclei of host cells, and also that conversion of the T-strand in a double stranded form is not affected by degradation of VirD2.

Discussion

A. tumefaciens induces tumor formation at wound sites in plants by transferring T-DNA into plant cells. VirD2 plays a key role in *Agrobacterium*-mediated T-DNA transfer; it participates in the entire transfer process, from the formation of single-stranded T-strand to the translocation of the T-complex through the T4SS into host cells. Inside the host cell it interacts with importin, which then mediates translocation of the T-complex into the nucleus and it may even be involved in the integration of the T-DNA into the chromosomal DNA. However, a putative role of VirD2 in the integration of the T-DNA into the host chromosomal DNA has remained obscure. In order to study the role of VirD2 inside the host cell nucleus, we exploited the auxin-induced degron (AID) system to destroy VirD2 once it enters the host cell nucleus. To this end, we constructed *Agrobacterium* strains expressing AID-tagged and native VirD2 and studied the effect of VirD2 degradation in the nucleus during the transformation of yeast and plants.

To test whether the AID-degron system could lead to degradation of VirD2 within the nucleus, we expressed an AID-tagged VirD2-GFP fusion protein in yeast strain DF5-TIR, in which the TIR1 F-box protein was expressed allowing auxin-induced activation of the SCF complex. As shown by confocal microscopy (Figure 2) the fluorescence of GFP-tagged VirD2 proteins largely disappeared upon auxin treatment in most cells. Also the fluorescence detected by flow cytometry was greatly reduced, indicating that VirD2 was degraded within the nucleus. On the other hand free GFP was not affected by the auxin treatment. This indicates that in yeast the AID system can be used to strongly reduce the levels of intracellular VirD2. Subsequently, we showed that the efficiency of AMT of yeast by an *Agrobacterium* strain expressing VirD2^{AID} was 10-fold reduced upon auxin-induced degradation of VirD2^{AID} (Figure 3), thus confirming that VirD2 plays a role in the transfer process inside the yeast cell.

With respect to plant cells, the size of the tumors induced by *Agrobacterium* strains expressing VirD2^{AID} was reduced compared to that of the tumors caused by strains expressing native VirD2. Besides, with the root transformation assay the number of calli formed by the strain expressing VirD2^{AID} was only 27% of the number of calli formed by the strain expressing native VirD2 (Figure 5B). These results confirm an important role of VirD2 in the transformation process inside plant cells. To investigate whether this lower efficiency is related to a reduced delivery of tagged VirD2 from *Agrobacterium* into plant cell nuclei, transient transformation assays using *Agrobacterium* strains carrying the binary vector pCAMBIA2301 were performed. The translocation of the T-DNA from *Agrobacterium* to the host cell nucleus was not affected by the AID tag on VirD2 (Figure 5C). Therefore, we can speculate that the observed lower stable transformation efficiency of strains with VirD2^{AID} is caused by degradation of VirD2^{AID} inside the nuclei of host cells rather than by a deficient translocation of the T-DNA from its entry point into the host cell to the cellular nucleus.

It has been shown that VirD2 can interact with histones in yeast (Wolterink-van Loo et al., 2015). In this way it may help direct the T-DNA to the chromatin as a prelude to integration into the host chromosomal DNA. In plants, it is possible that VirD2 interacts with histone proteins or other chromatin factors to drive T-DNA to plant chromatin regions (Gelvin and Kim, 2007), facilitating the integration process. In agreement with this, the local chromatin structure may affect AMT. Overexpression of specific histone proteins increased AMT efficiency in plants (Mysore et al., 2000; Tenea et al., 2009) and deletion of histone-related genes altered AMT efficiency in yeast (Soltani et al., 2009). Taken together, we can speculate that VirD2 can interact with host factors to participate in the integration process as well.

In conclusion, using the degron approach developed in this chapter we found that the efficiency of AMT goes strikingly down after degradation of VirD2 even though the T-DNA was delivered successfully into the nucleus of the host cell. Although direct evidence for a role of VirD2 in the integration process is still lacking, it can be concluded that VirD2 has a role inside the nucleus of the host cell to promote stable transformation. We have to be careful with this conclusion though, because we have not added auxin in the plant experiments and our results may therefore have been influenced by local differences in auxin concentration. Another pitfall, which we have not addressed so far, may be that the SCF-TIR1 complex may be functional to some extent also in the cytoplasm. Further experimentation will have to be done to address these issues.

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Table 1. Yeast and *Agrobacterium* strains used in this study

Yeast strain	Genotype	Source/Reference
BY4741	MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Brachmann et al., 1998
BY4741 <i>ada2Δ</i>	BY4741 <i>ada2::hphMX4</i>	Chapter 2
DF5	MATa <i>his3Δ200 leu2Δ3,2Δ112 lys2Δ801 trp1Δ1 ura3Δ52</i>	Finley et al., 1987
DF5-TIR1	DF5 URA3:: ADH1-AfTIR19myc	Morawska and Ulrich, 2013
DF5-TIR1-pUG34	TIR1 with plasmid pUG34	This study
DF5-TIR1-pSDM4695	TIR1 with plasmid pSDM4695	This study
DF5-TIR1-pSDM4696	TIR1 with plasmid pSDM4696	This study
DF5-TIR1 <i>ada2Δ</i>	DF5-TIR1 <i>ada2::hphMX4</i>	This study
<i>Agrobacterium</i> strain	Description	Source/Reference
LBA1100	<i>Agrobacterium</i> strain C58 cured, pTiB6, ΔT -DNA, Δocc , Δtra , Rif, Spc	Beijersbergen et al., 1992
LBA1010	<i>Agrobacterium</i> strain C58 cured, pTiB6, Rif	Koekman et al., 1982
LBA2556	LBA1100 <i>virD2Δ</i> , Rif, Spc	Jurado-Jacome, 2011
LBA2569	<i>Agrobacterium</i> strain LBA1010 <i>virD2Δ</i> , Rif, Spc	van Kregten, 2011
LBA1100-pSDM8001	LBA1100 with binary vector pSDM8001, Km	van Attikum and Hooykaas, 2003
LBA1100-pCAMBIA2301	LBA1100 with binary vector pCAMBIA2301, Km	This study
LBA2556-pSDM3149-pSDM8001	LBA2556 with binary vector pSDM3149 and pSDM8001	This study

LBA2556-pSDM3149- pCAMBIA2301	LBA2556 with binary vector pSDM3149 and pCAMBIA2301	This study
LBA2556-pSDM4699- pSDM8001	LBA2556 with binary vector pSDM4699 and pSDM8001	This study
LBA2556-pSDM4699- pCAMBIA2301	LBA2556 with binary vector pSDM4699 and pCAMBIA2301	This study

Table 2. Plasmids used in this study

Plasmid	Specifications	Source/Reference
pSDM3149	pRL662, pvirD-virD2	Jurado-Jacome, 2011
pHyg-IAA ¹⁻¹¹⁴ -8myc	N-terminal tagging signal of IAA17 in parent vector pSM409	Morawska and Ulrich, 2013
pSDM4694	pHyg-AID ⁷¹⁻¹¹⁴ -8myc	This study
pSDM4697(VirFCT)	pHyg-IAA ¹⁻¹¹⁴ -VirFCT-8myc with C-terminal of VirF	This study
pSDM4694(VirFCT)	pHyg-AID ⁷¹⁻¹¹⁴ -VirFCT-8myc with C-terminal of VirF	This study
pSDM4698	pRL662, pvirD-virD2 without stop codon	This study
pSDM4699	pRL662, pvirD-VirD2 ^{AID}	This study
pSDM4700	pRL662, pvirD-VirD2 ^{IAA}	This study
pUG34	Centromeric plasmid with a <i>HIS3</i> marker to express N-terminal GFP fusions under the control of the <i>MET25</i> promoter	Güldener et al., 1996
pRUL1146	pUG34-virD2	Soltani, 2009
pSDM4695	pUG34-VirD2 ^{IAA}	This study
pSDM4696	pUG34-VirD2 ^{AID}	This study
pSDM8001	<i>Agrobacterium</i> binary vector with KanMX selectable marker and <i>PDA1</i> flanking sequences	van Attikum and Hooykaas, 2003
pCAMBIA2301	<i>Agrobacterium</i> binary vector with NeoR/KanR selectable marker and GUS genes	Hajdukiewicz et al., 1994
pGPINTAM	Binary vector with a tamoxifen-inducible promoter	Friml et al., 2004

Table 3. Primers used in this study

Primer name	Sequence (5'-3')	Restriction enzyme
VirFCT-Fw	CCCCC <u>GGGCTCGAGG</u> TATGGCAGAAGTTC	<i>XmaI</i> - <i>XhoI</i>
VirFCT-Rev	CCCCC <u>GGGGACGAACAGCACGG</u> ATAGTC	<i>XmaI</i>
AID-Fw	TT <u>GGTACCCCTAAAGATCCAGCCAA</u> ACC	<i>KpnI</i>
AID-Rev	CCCCC <u>GGGTGATACCTT</u> CACGAACGCCGC	<i>XmaI</i>
VirD2-Fw	GCGGCCGCT <u>CTAGAGG</u> ATCC	<i>XbaI</i>
VirD2-Rev	CGGAATTCGCGCCCATCGTCGCGACGAT	<i>EcoRI</i>
IAA-Fw	CGGAATTCGCTACGCTGCAGGTCGAC	<i>EcoRI</i>
IAA-Rev	CGGAATTCGACGAACAGCACGGATAG	<i>EcoRI</i>
ADA2-Fw	TAAATATACAGCGTAGTCTGAAAATATATACATTAAG CAAAAAGACAGCTGAAGCTTCGTACGC ATAATAACTAGTGACAATTGTAGTTACTTTTCAATTTT	
ADA2-Rev	TTTTTTGCCGCGGCCGCATAGGCCAC	

Restriction sites are underlined.

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